Limnologica 58 (2016) 7–10

Contents lists available at ScienceDirect

Limnologica

journal homepage: www.elsevier.com/locate/limno

Shared microsporidian profiles between an obligate (*Niphargus*) and facultative subterranean amphipod population (*Gammarus*) at sympatry provide indications for underground transmission pathways

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ARTICLE INFO

Article history:
Received 3 September 2015
Received in revised form 27 January 2016
Accepted 28 January 2016
Available online 1 February 2016

Keywords:
Cave ecology
DNA barcoding
Microsporidia
Host-parasite interactions
Stygofauna
Groundwater

ABSTRACT

Only sparse knowledge exists on amphipod-infecting microsporidians in subterranean waters. Here, we DNA barcoded two sympatric amphipod populations (obligate subterranean *Niphargus schellenbergi*, facultative *Gammarus fossarum*) and their microsporidian parasites. Parasite prevalence was assessed by diagnostic PCR assays. Overall prevalence was 82.7 %. Both amphipod populations shared all four identified parasite species (*Nosema granulosis*, *Microsporidium* sp. I, *Orthosomella* sp., *Microsporidium* sp. BPAR3). Hence, we postulate underground transmission pathways of microsporidians mediated by subterranean amphipod hosts.

Main text

Microsporidians are reduced, unicellular endoparasitic fungi-relatives frequently found in both, invertebrate and vertebrate species. Their mode of transmission can be vertical (i.e. within the same host species), horizontal (i.e. between different host species) or both (Stentiford et al., 2013). The diversity of microsporidians and their effects on amphipod crustaceans – ecological keystone species in many surface and subsurface freshwater ecosystems (MacNeil et al., 1997) – are permanent targets of parasitological research (e.g. Bulnheim, 1975; Krebes et al., 2010). It has been demonstrated that microsporidians can alter the behaviour and ecological tolerances of their amphipod hosts or lead to feminization of the population (Kelly et al., 2002; Stentiford et al., 2013). Up to now, research has primarily focussed on surface amphipod freshwater populations and almost no attempt has been made to assess microsporidian diversity and their prevalences in subterranean communities. It has to be highlighted that Ford and Glazier (2008) explicitly investigated a population of *Gammarus minus* at Falling Branch Cave (Virginia, USA) for microsporidian parasites. This population largely comprised females demonstrating intersex characteristics, i.e. a known consequence of feminising microsporidians. Nevertheless, they found no indication of microsporidian spores by a morphological investigation of several specimens. Other studies reported microsporidians from groundwaters (Dowd et al., 2003; Kamper, 1997), but no direct connections to amphipods have been made. Only Poisson (1924) described two microsporidium species, *Mrazekia niphargi* and *Thelohania vandeli*, infecting the stygobiont amphipod *Niphargus stygius* in the vicinity of Paris (France). Later, *T. vandeli* was removed from the genus *Thelohania* in the course of a revision by Hazard and Oldacre (1976) and placed into the holding genus *Microsporidium*, an artificial group used for so far unclassified microsporidia (Vossbrinck et al., 2014). Similarly, Vandel (1964) treated *M. niphargi* as a *nomen nudum*. Yet, and to our knowledge, the two infections reported by Poisson – by *Microsporidium niphargi* and *Microsporidium vandeli* – are the only two published cases of microsporidians infecting subterranean amphipods.

Our aim here is to study the microsporidian diversity and prevalences between an obligate (*Niphargus*) and facultative subterranean (*Gammarus*) amphipod population at sympatry. Amphipods of the genus *Gammarus* are infected by a number of

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http://dx.doi.org/10.1016/j.limno.2016.01.005
0075-9511/

Table 1
Universal and specific primers* designed to detect microsporidian species.

Primer	Sequence (5′–3′)	Annealing temperature (°C)	Specificity
V1	CAC CAG GTT GAT TCT GCC TGA C	68	Universal (Zhu et al. (1993))
Mic-uni3R	ATT ACC GCG GMT GCT GGC AC		Universal (this study)
Ortho F	CAC GAT AAC CAC GGG AAA CTG TG	68	*Orthosomella* sp. (this study)
Mic-uni3R	ATT ACC GCG GMT GCT GGC AC		
N_gran F	CTT AAC AAG ACT ATG ACG GAT A	60	*Nosema granulosis* (this study)
N_gran R	TCA TCC AGT TAG GGT TAT CAC A		
Mic7 F	ACA GTT ATA ATT TAC TCG TAG ATC	58	*Microsporidium* sp. BPAR3 (Grabner et al. (2015))
Mic7 R	TAC TCG CAA GCA TGT GCT CA		
Mic3 F	CAG TAA TGT TGC GAT GAT TTG GTC	58	*Microsporidium* sp. I (Grabner et al. (2015))
Mic3 R	CAG TAA ATA CTC CAC AGT ATC TTA C		

* Targeting the small subunit ribosomal DNA, PCR program for V1-Mic-uni3R: 94°C–3 min, 40 cycles of 94°C–30 s and 68°C–35 s and final elongation at 68°C–3 min. For all other primers: 94°C for 3 min, 40 cycles of 94°C–30 s, [annealing temp. see table]–30 s, 68°C–35 s and final elongation at 68°C–3 min.

microsporidian species, some demonstrating rather low host specificity (e.g. *Microsporidium* sp. I, *Nosema granulosis*) (Grabner et al., 2015; Krebes et al., 2010). Since it is known that *Gammarus* sp. can temporarily or permanently live in subterranean freshwaters (Carlini et al., 2009; Flot and Weber, 2013), it is likely that some of their associated (horizontally transmitted) microsporidians may be shared with the subterranean *Niphargus* community. On the other hand, *Niphargus* specimens frequently occur outside of subterranean habitats (by active migration at night or passive transportation during floods) and thus, may encounter surface populations (Luštrik et al., 2011). Hence, an exchange of microsporidians seems plausible, e.g. by horizontal transmission via predation of infected specimens, feeding on carrion or oral digestion of spores in co-inhabited waterbodies (Fišer et al., 2010). Here, we explicitly want to test, whether both amphipod populations share microsporidian species or demonstrate explicit pathogen profiles.

Amphipod crustaceans in general, but *Gammarus* and *Niphargus* in particular, are prone to comprise cryptic species, e.g. see Weiss et al. (2014) for an example on *Gammarus fossarum*. Moreover, microsporidian infections may be misclassified or even completely overlooked by morphological investigations. To circumvent these problems and to unambiguously identify host and parasite species, we used the approach of DNA barcoding. Microsporidian prevalences were assessed by a diagnostic PCR screening assay with newly designed primers (Table 1).

Our sampling site was the Tunnel of Huldange (synonyms: Tunnel of Lengeler, Tunnel of Wilwerdingen) in the North of Luxembourg, constituting an old railway construction with a length of approx. 790 m (Fig. 1A). The tunnel crosses several water veins and two small slow-flowing streams exit in a northward and southward direction, respectively (Fig. 1). At this artificial subterranean site three amphipod species occur in sympatry (Flot and Weber, 2013): *Gammarus pulex* Linnaeus, 1758 and *G. fossarum* Koch, 1836, both inhabit small ponds at the entrance zones, but are occasionally found inside the tunnel. *Niphargus schellenbergi* Karaman, 1932, a common species in Northern Europe and obligate cave-dweller (Fig. 2B), is frequently observed in the dark zone of the tunnel, but some specimens are found under stones at the north entrance. At seasons with greater water flow, we can expect some specimens to be washed out in both directions.

On 31/10/2014, 31 specimens of *Gammarus* and 21 specimens of *Niphargus* were collected by hand and immediately stored in 96 % ethanol. *Niphargus* specimens originated from the dark zone of the tunnel and from stones close to the north entrance (Fig. 1B and C). *Gammarus* specimens were separately collected either outside (20 individuals) at the south entrance (G–o, Fig. 1D) or inside (11 individuals) within the dark zone along the southern half of the tunnel (G–i). No *Gammarus* specimens were observed along the northern half of the tunnel or in the pond at the north entrance.

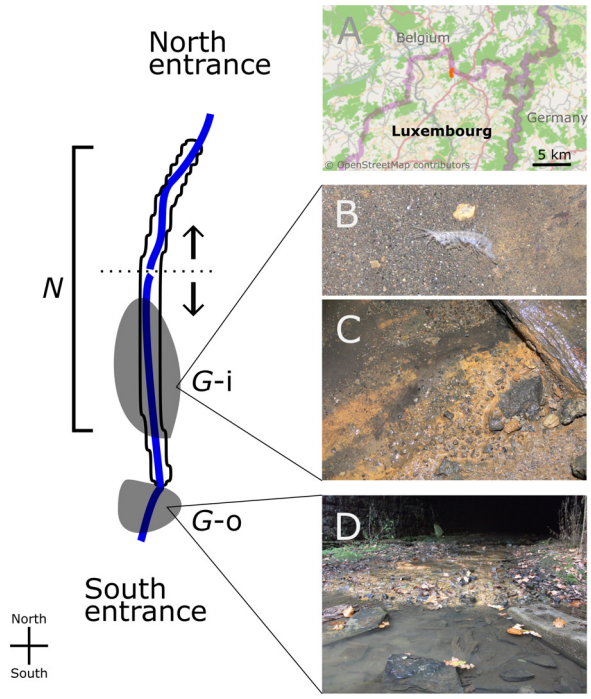

Fig. 1. Schematic overview of the Tunnel of Huldange. N: collection site of *Niphargus* specimens within the tunnel; G–i: collection site (grey shaded) of *Gammarus* specimens within the tunnel; G–o: collection site (grey shaded) of *Gammarus* specimens outside the tunnel. Dotted line indicates the approximate location where flow direction of the water changes. (A) Location of the Tunnel of Huldange (marked in red) in northern Luxembourg, close to the border with Belgium. (B) Specimen of *Niphargus schellenbergi*. (C) *Niphargus* (N) and *Gammarus* habitat (G–i) within the tunnel. (D) South entrance and *Gammarus* habitat (G–o) outside the tunnel.

Whole amphipod specimens were used without dissection for DNA isolation, DNA barcoding of the hosts and to test for prevalences of microsporidians. DNA isolation and DNA barcoding were performed according to Grabner et al. (2015). The standard DNA barcoding locus, i.e. the cytochrome c oxidase subunit 1 (CO1), was amplified for five amphipod specimens of each site. PCR products were purified using a JETQUICK PCR Product Purification Spin Kit (Genomed) according to manufacturer's instructions and were bi-directionally sequenced at GATC Biotech AG (Köln, Germany).

To test for microsporidian infections, all samples were screened by diagnostic PCRs according to Grabner et al. (2015). All Microsporidian-PCR reactions were performed with the AccuStart II PCR ToughMix (Quanta Bioscience). One reaction contained 10 μL of 2 × ToughMix, 0.5 μM of each primer and 1 μL of DNA. Water was added up to a total volume of 20 μL. PCR conditions for all primers are shown in Table 1.